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Inhibitory effect of *Lactobacillus plantarum* isolated from intestinal microbiota on *Clostridium difficile* infection

장 내 균총에서 분리 한 *Lactobacillus plantarum*의 *Clostridium difficile* 감염 저해 효과

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Abstract

Inhibitory effect of *Lactobacillus plantarum* isolated from intestinal microbiota on *Clostridium difficile* infection

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Clostridium difficile infection (CDI) is one of the most widely known nosocomial infections. The dysbiosis of gut microbiota due to antibiotic use is the leading cause of CDI; people with relatively healthy gut microbiota do not typically become infected with CDI. It is, therefore, necessary for researchers to find a specific bacterium that inhibits CDI among healthy gut microbiota to regulate it. However, there have been few studies about CDI-resistant strains among healthy gut microbiota. Therefore, this study aims to verify the inhibitory effect of single strains isolated from normal individuals on CDI and to determine the underlying mechanism. Through previous studies, three strains isolated from healthy Koreans with *C. difficile* inhibitory effects were selected. The CDI

inhibitory effects of the three strains were confirmed through animal experimentation. CDI inhibition was assessed by post-infection weight change, *C. difficile* colony forming unit (c.f.u.), and the toxin A&B measured value. The feces collected in the animal experiment were used to analyze the changes and status of gut microbiota and gut metabolome. As a result of this experiment, a significant inhibition of CDI was observed in the group to which *Lactobacillus plantarum* SNUG 10271 was administered. Gut microbiota analysis showed that *Ruminococcaceae* and *Coprococcus* showed high abundance in the *L. plantarum* SNUG 10271 group and was negatively correlated with *C. difficile*. Gut metabolome analysis showed that the concentration of deoxycholic acid (DCA) was significantly higher in individuals with inhibitory effects. These results confirmed the possibility of CDI inhibition of a single strain isolated from healthy individuals. We also found information on *Ruminococcaceae*, *Coprococcus*, and DCA that will be necessary to understand the mechanism of CDI inhibition.

Keywords: *Clostridium difficile* infection, Microbiota, *Lactobacillus plantarum* SNUG 10271, *Ruminococcaceae*, *Coprococcus*, *Deoxycholic acid*

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I. Introduction

Clostridium difficile infection (CDI) is a bacterial infection caused by the Gram-positive, spore-forming bacterium *Clostridium difficile* [1]. *Clostridium difficile* infects the gastrointestinal tract, leading to colonocyte death, loss of intestinal barrier function, watery diarrhea and pseudomembranous colitis [2], [3]. The continued use of antibiotics during hospitalization and the collapse of the healthy gut microbiota are considered to be major risk factors for CDI outbreaks [4], [5]. CDI is a worldwide disease [6]. According to the Centers for Disease Control and Prevention (CDC), CDI has caused 14,000 deaths each year in the United States and costs more than $1 billion in medical expenses [7]. In Korea, the incidence of CDI increased from 1.9 per 10,000 in 1998 to 8.82 in 2006–2007 [8]. For this reason, CDI-related prevention and treatment has been extensively studied around the world [5]. It has been confirmed that dysbiosis of intestinal microbiota plays a pivotal role in not only the severity of CDI symptoms but also the outbreak of CDI [9].

CDI begins by germinating in the gastrointestinal tract after the introduction of *C. difficile* spores through the oral cavity [10]. After

spore germination, *C. difficile* causes symptoms by producing homologous toxin A and toxin B (toxin A&B) [11]. Toxin A&B binds to the epithelial cell surface and is internalized by endocytosis [12]. The glucosylation of the Rho family GTPases at the cell membrane by toxin A&B results in the inactivation of actin cytoskeleton intestinal epithelial cells [13]. In this case, the tight junction of the intestinal epithelial cells is loosened, and consequently, the gut permeability is increased along with the influx into the host of intestinal microbes, including *C. difficile* [14]. As a result, intestinal cell destruction leads to diarrhea and weight loss, and in severe cases, symptoms such as a megacolon or even death may occur [15].

Secondary metabolites produced by commensal bacteria are known to play an important role in the germination and growth of *C. difficile* together with gut microbiota [16], [17]. Typically, secondary bile acid, deoxycholic acid (DCA), is known to inhibit *C. difficile* growth [18], [19]. Methylcholate is known to inhibit the binding of toxin A & B to the cell surface [20], and n-butyrate is known to regulate intestinal cell regeneration and immune response [21]. Therefore, to regulate CDI, it is necessary to understand the secondary metabolites produced by the commensal bacteria together with the gut microbiota

[22].

People with healthy gut microbiota are resistant to CDI [23]. However, specific components of the healthy gut microbiota that are resistant to CDI have pivotal functions that have not been completely investigated. Therefore, identifying specific strains that have CDI inhibitory effects in the healthy gut microbiota is necessary to solve the problem caused by CDI [24]. Understanding the link between commensal microbiota and *C. difficile* through these studies may contribute to preventing CDI-related complications such as the development of a megacolon [25]. This study aimed to identify the strains with CDI inhibitory effects and to find the factors associated with CDI inhibition by analyzing gut microbiota and gut metabolites.

In previous studies, three strains (*Lactobacillus plantarum* SNUG 10271, *Bacteroides ovatus* SNUG 40239, and *Clostridium scindens* SNUG 40402) that inhibited the growth of *C. difficile* vegetative cells through an *in vitro C. difficile* inhibition assay were selected [41]. In this study, the CDI inhibitory effect of each strain was confirmed by the widely used CDI animal experimental model [26]. To find the clues necessary to reveal the mechanism of inhibition, five time-points were set throughout the experimental process to analyze gut

microbiota and gut metabolome (Fig. 1).

Taken together, we verified the *in vivo* CDI inhibitory effect of *L. plantarum* SNUG 10271. Also, it was confirmed that the abundance of specific bacterial taxa, such as *Ruminococcaceae* and *Coprococcus,* and the concentration of DCA were increased in the group with a CDI inhibitory effect.

II. Materials and Methods

1. Culture condition of bacteria

Bacteroides ovatus SNU 40239 (*B. ovatus* SNUG 40239), *Clostridium scindens* SNUG 40402 (*C. scindens* SNUG 40402) and *Lactobacillus plantarum* SNUG 10271 (*L. plantarum* SNUG 10271) isolated from Korean feces and *Clostridium scindens* KCTC 5591 (*C. scindens* KCTC 5591) (Type strain) were used as CDI inhibitory strains. *B. ovatus* SNU 40239 was cultured in brain heart infusion medium supplemented with 5% fetal bovine serum (BHIF). *C. scindens* SNUG 40402 and *C. scindens* KCTC 5591 were cultured in Gifu Anaerobic Broth (GAM). *L. plantarum* SNUG 10271 was cultured in Man Rogosa Sharpe (MRS). Since all strains were anaerobic bacteria, they were inoculated in an anaerobic chamber (Coy, USA) containing a mixture of 5% H2 and CO2 in N2, as described previously with some modifications[27] and incubated at 37 ° C for 24 h. Single colonies were picked using sterile loops and inoculated into broth media and activated twice. Finally, 10^9 cells were used for animal experiments.

2. Sporulation of *C. difficile*

Clostridium difficile ATCC 43255 (*C. difficile* ATCC 43255) is adjusted to an OD value of 0.2 in broth and 100ul of *C. difficile* ATCC 43255 is plated on SMC medium (90 g Bacto peptone, 5 g protease peptone, 1 g NH4SO4, 1.5 g Tris base, and 15 g agar per liter) [28]. After culturing at 37 ° C for seven days, all of the colony is scraped off and suspended in 1 ml of cold 1x PBS in a 1.7 ml tube. Store in this state at 4 ° C for 24 h. Spin down at 14000 rpm for 1 minute. Remove supernatant, wash with 1 ml of cold PBS, and spin down at 14000 rpm for 1 minute. After removing the supernatant, add 1 ml of 70% ethanol and store for 1 h. Spin down at 14000 rpm for 1 minute. Remove the supernatant and wash with 1 ml of sterile PBS. Store at -80°C until use.

3. Murine *C. difficile* infection time-course experiment

Mouse experiments were performed with C57BL/6 female 6-week old mice purchased from Orient Bio Inc. (Seongnam, Korea). All mice were maintained in a specific-pathogen-free facility at Seoul National University Hospital Biomedical Research Institute. Animal experiments were performed following Seoul National University Hospital Biomedical Research Institutional guidelines and approved by the institution's Institutional Animal Care and Use Committee (IACUC).

Five mice were randomly assigned to each cage in consideration of body weight and standard deviation, and the experiment started after a week of stabilization.

Animal experiments were conducted in seven groups. Three group which are administered CDI inhibitor strain (*Bacteroides ovatus* SNU 40239, *Clostridium scindens* SNUG 40402 and *Lactobacillus plantarum* SNUG 10271), Mix group with three CDI inhibitor strains at the same ratio, PBS positive control group, and Non-infected negative control group. Also, to measure the degree of the effect of the inhibitory strains, a total of seven groups including *Clostridium scindens* KCTC 5591, which has been proven to be effective, were tested [18].

Animal experiments followed the most widely used conventional methods [26]. 7 week old mice were fed water containing an antibiotic cocktail containing kanamycin (0.4 mg/mL), gentamicin (0.035 mg/mL), colistin (850 U/mL), metronidazole (0.215 mg/mL), and vancomycin (0.045 mg/mL) for 3 days. After replacing the antibiotic cocktail with normal drinking water, four inhibitory strains (10^9 CFU/mouse) were orally administered at 24 h intervals over the course of two days. 24 h after the final oral administration, 200 μg of

clindamycin was intraperitoneally (IP) injected. Finally, *Clostridium difficile* ATCC 43255 spores (10^4) were administered orally 24 h after the administration of clindamycin IP. Weight loss and mortality post-infection were measured at 24 h intervals over the course of eight days.

To investigate microbiome and metabolome changes, we conducted mouse fecal sampling at five time-points. Fecal samples were collected at the following time points: at the pre-antibiotic treatment (day 0), post-antibiotic treatment has concluded (day 3), after treatment with candidate inhibitory strains (day 5), after Clindamycin IP injection (day 6) and *C. difficile* spore infection (day 7). Fecal samples were collected in a sterile tube on a clean bench to prevent contamination and stored at -80°C until analysis.

4. Measurement of *C. difficile* c.f.u. and toxin A&B

Feces were weighed and dissolved in 1 ml PBS buffer in an anaerobic chamber. The diluted solution was then inoculated on *C. difficile* selective (agar chromID® Culture Media) (bioMérieux, USA) and incubated at 37°C for 48 hr. After counting, the number of cells was corrected by considering the weight of the stool. *C. difficile* toxin A and B were quantified using *C. difficile* TOX A/B II ELISA kit

followed the product manuscript. (Techlab, USA).

5. Fecal microbiota analysis

The bacterial DNA contained in the fecal samples collected at the aforementioned time points were extracted using QIAamp DNA Stool Mini Kit according to the manufacturer's instruction (Qiagen, Germany). The V4 region of the 16S rRNA gene in the extracted fecal DNA was amplified with the Illumina-adapted universal primers 515F/806R [29]. The amplified PCR products were purified using Qiagen PCR clean-up Kit (Qiagen, Germany). Then, the concentrations of the amplified PCR products were measured using the KAPA Library Quantification Kit (KAPA biosystems, USA) and ABI 7300 (Applied Biosystems, USA). Finally, the quantified PCR products were pooled after calibrating the concentration constant and sequenced using the Miseq platform (Illumina, USA).

Sequence data was analyzed using a Quantitative Insights into Microbial Ecology (QIIME) platform (ver. 1.8.0) and clustered into 97% identity using a closed method operational taxonomic unit (OTU) picking method [30]. After alignment, OTU were classified using the Ribosomal Database Project (RDP) classifier based on the Greengene

database. It was analyzed to the species level based on the OTU table and relative abundance calculated.

Beta diversity between each group and time point was analyzed using Unifrac distance. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to find bacterial biomarkers among different groups [31]. Finally, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database-based Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was conducted to investigate the differences in bacterial function among the microbiomes of the different mouse groups [32].

6. Quantification of DCA and metabolome analysis of fecal samples

Feces were weighed, and 80% methanol was added at 1: 1 ratio to extract bile acids and metabolites in feces. After sonication for 3 min and centrifugation at 13,000 rpm for 1 min, the supernatant was filtered with 0.22 μm filter. The filtered fecal metabolite solution was then evaporated in Speedback (Eppendorf, Germany), after which metabolites contained within the fecal sample, including bile acid, were obtained in a powder and stored at -80°C until analysis [18].

Bile acids (TCA, CA, CDCA, LCA, DCA) separation was performed using an Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 m) and Acquity UPLCTM system (Waters). The analysis conditions for chromatography were as follows: mobile phase A, water with 0.1% formic acid; mobile phase B, acetonitrile with 0.1% formic acid; injection volume, 2 μl. The time gradient of mobile phase B was from 5% to 90% in 15 min, and the flow rate was 0.4 ml/min. Qualitative bile acids analysis was conducted using Waters Synapt G2-Si Q-TOF mass spectrometer (Waters) equipped with an electrospray (ESI) probe and negative ionization mode with the tofMRM mode. For the Synapt G2-Si Q-TOF, the following mass spectrometer parameters were applied: capillary, 25kV; source temperature, 100°C; sampling cone, 40; source offset, 80; desolvation temperature, 250°C; cone gas flow, 50 L/h; desolvation gas flow, 600 L/h; nebuliser gas flow, 6.5 Bar. The measured bile acid was quantified using QuanLynx of MassLynx program [33].

Supernatant metabolite measurement was performed in positive ionization mode and MS^e scan mode using the same column and UPLC conditions. The mass range was set from 50 to 1200 Da and the scan time was set to 0.2 sec. The following mass spectrometer parameters

were used: capillary, 2 kV; source temperature, 120°C; sampling cone, 40; source offset, 80; desolvation temperature, 400°C; cone gas flow, 50 L/h; desolvation gas flow, 600 L/h; nebuliser gas flow, 6.5 bar. All systems were controlled by Mass-LynxTM software 4.1 (Waters) [33].

Intergroup metabolism analysis was carried out by transferring MassLynxTM software to the Progenesis QI software (Waters). A retention time window of 0.20 min and mass tolerance of 1.0 ppm were set to align the compounds. Then, ANOVA p-value and max fold change were applied to filter compounds and then exported to EZinfo software (version 3.0.3.0) for principal component analysis (PCA) [34].

7. Statistical analysis

All statistical analysis was performed using Prism 7 (GraphPad Software, USA). Statistical significance was measured using Mann-Whitney test to analyze differences between two groups with similar variances and the Kruskal–Wallis test with Dunn correction for multiple comparisons was used when three or more groups were measured with similar variances. In all graphs, data were presented as mean + standard error of mean (SEM). Statistical significance was given as * P-value < 0.05, ** P-value < 0.01, *** P-value < 0.001.

III. Results

1. *L. plantarum* SNUG 10271 inhibit clinical symptoms of CDI

After inducing CDI, changes in symptoms between groups were observed to evaluate the effect of CDI inhibitory strains. The priority factors in determining the severity of CDI symptoms, weight loss, and mortality, were monitored for eight days after CDI. As a result, a significant change in body weight was not observed for 24 h after the infection, but the weight of all groups decreased rapidly from 24 h to 72 h (Fig. 2a). However, among the five groups (*L. plantarum* SNUG 10271, *B. ovatus* SNUG 40239, *C. scindens* SNUG 40402, *C. scindens* KCTC 5591, Mix) treated with the distinct inhibitory strain prior to infection, only the *L. plantarum* SNUG 10271 group lost less than 7% on average of their weight (Fig. 2b, c). Compared to other groups, this inhibition of weight loss was significant at 48 and 72 h after CDI (Fig. 2b, c).

The mortality rate was also observed consistent with weight loss. The first deaths occurred in the Mix group at 48 h after infection, when rapid weight loss occurred (Fig. 3a). After that, death occurred in all groups except the *L. plantarum* SNUG 10271 group and the Non-

infection group for six days after the infection (Fig. 2a). To summarize, only one mouse died within the *C. scindens* SNUG 40402 group, the Mix group and the *C. scindens* KCTC 5591 group, and four mice died in the *B. ovatus* SNU 40239 group (Fig. 3a). Three mice died in the PBS group (Fig. 3a).

The feces gathered at 24 and 48 h following infection was used to measure the abundance of *C. difficile* c.f.u. and toxin A&B titer responsible for the severity of CDI symptoms. Unlike weight loss and mortality, there was no significant decrease in *C. difficile* c.f.u. at 24 and 48 h after CDI (Fig. 3c, d). Also, there was no significant statistical difference in toxin A&B titer between groups (Fig. 3b). However, a tendency for lower toxin A&B titer was observed in the *L. plantarum* SNUG 10271 group (Fig. 3b).

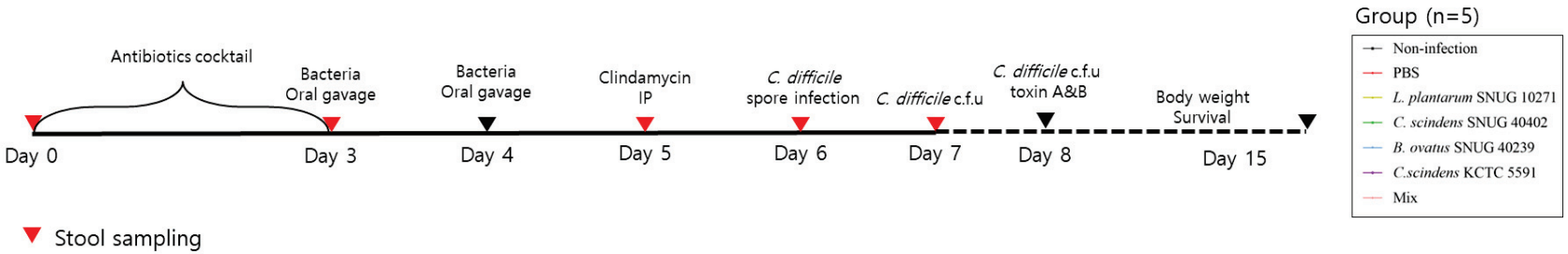

Figure 1. CDI scheme for C57BL/6 mice.

Mouse experiment was started with C57BL/6 mice by administering an antibiotic cocktail daily for 3 days. After antibody cocktail administration, isolated bacteria strains (10^9) were orally administered daily on day 3 and 4. Clindamycin is IP injection at day 5. CDI was induced by oral administration of *C. difficile* spore (10^6) at day 6. Weight change and mortality were measured for 8 days daily. The experiment was conducted into seven groups. Stool sampling was performed at the time indicated by red color. Mix: three strains (*L. plantarum* SNUG 10271, *C. scindens* SNUG 40402, *B. ovatus* SNUG 40239) were fed in equal proportions.

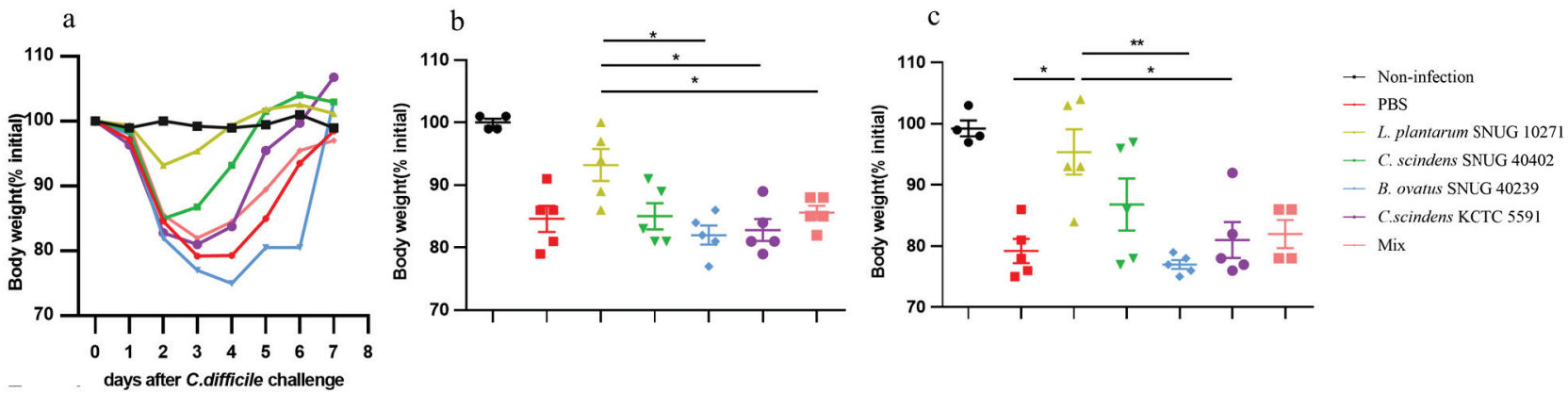

Figure 2. Mice body weight change after CDI

All body weights were converted to % based on infectious body weight (n = 5 per group). (a) The change in body weight of the whole group for eight days after CDI. (b) The difference in body weight between groups after 48 h after CDI. (c) The difference in body weight between groups after 72 h after CDI. * : P<0.05, ** : 0.01. (unpaired Mann–Whitney U-test).

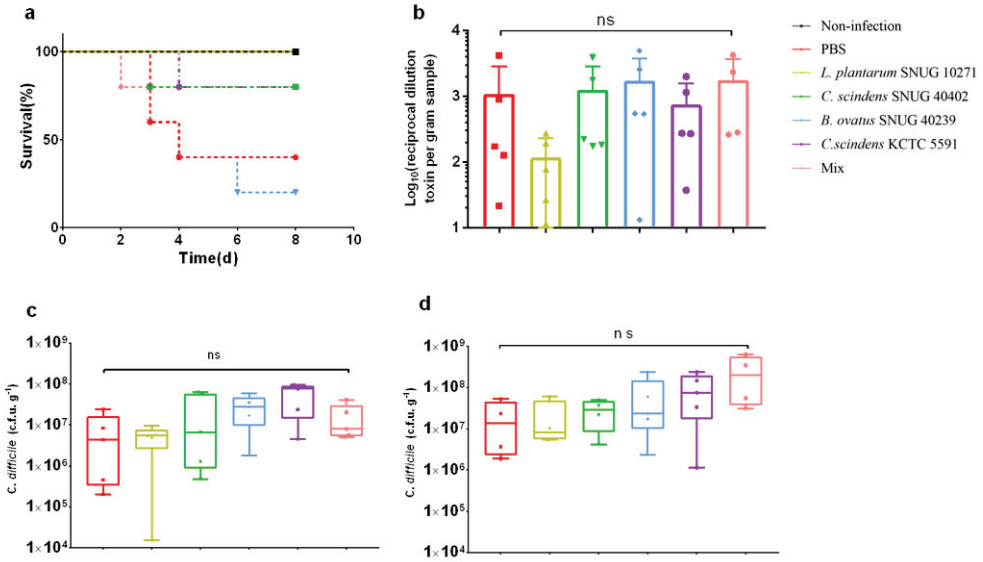

Figure 3. Mice survival change and measurement of *C. difficile* c.f.u. and toxin A&B

Survival change after CDI and *C. difficile* c.f.u. and toxin A&B in the feces acquired from 24 h and 48 h after CDI (n = 5 per group). (a) The survival change from the date of infection. (b) Toxin A & B measurements in feces 48 h after CDI. Measured *C. difficile* (c.f.u./g) in the feces (c) 24 h and (d) 48 h after CDI. * : P<0.05, ** : 0.01. (unpaired Mann–Whitney U-test).

2. Comparison of gut microbial diversity

Gut microbial diversity analysis was performed to find the difference of gut microbiota structure associated with CDI inhibition effect of the administered bacteria. Alpha diversity was analyzed using the Chao 1 index to measure species richness in the group. Also, non-metric multidimensional scaling (NMDS) for beta diversity was conducted to determine whether there is a difference in species diversity between groups. As a result of the alpha diversity measurements, species richness was lower in all groups after antibiotic administration, as expected (Fig. 4a). This species richness did not recover to pre-antibiotic (Day 0) levels at the time point when the CDI inhibitory effect was observed in the *L. plantarum* SNUG 40329 group (Day7). (Fig. 4a). While, the *B. ovatus* SNUG 40239 group, which showed the most severe symptoms after CDI, showed a more significant decrease in species richness after clindamycin IP than the Non-infection group did (Fig. 4a). Also, it was confirmed that the species richness in all groups after antibiotic treatment did not recover to the species richness present before antibiotic treatment (Fig. 4a).

Beta diversity analysis was conducted to determine whether species diversity between groups was related to CDI inhibition when

there was no inter-group difference in species richness. However, the clustering of the *L. plantarum* SNUG 10271 group distinct from other groups was not observed (Fig. 4b). As a result, the Mix group and the *B. ovatus* SNUG 40239 group were clustered into one group, and the remaining groups were clustered together (Fig. 4b). That is, there was no significant intergroup species richness and diversity in each separate group.

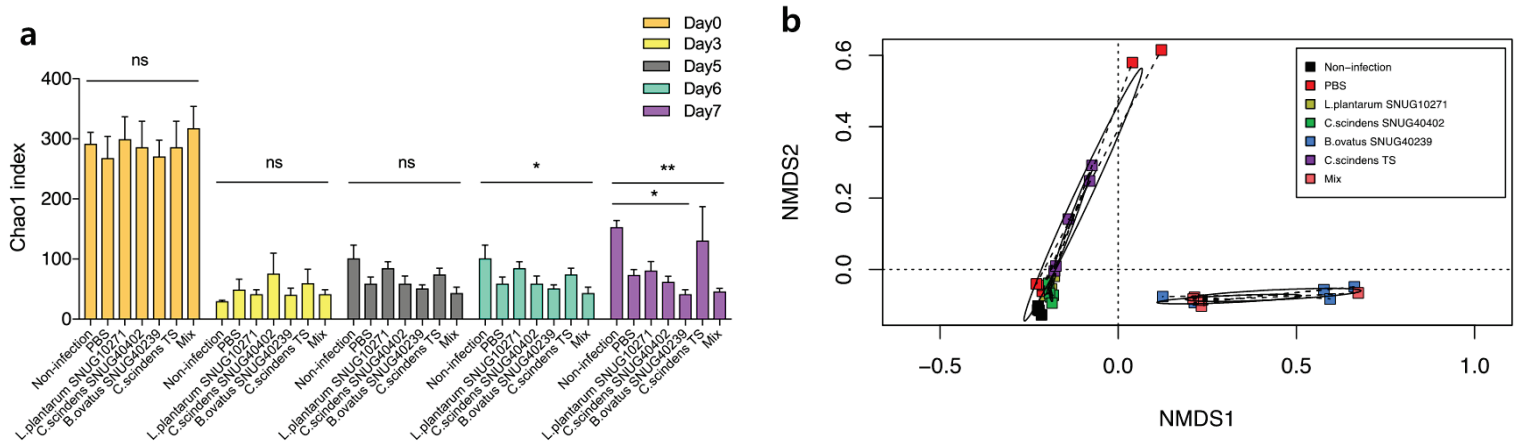

Figure 4. The alpha and beta diversity of the gut microbiota

(a) The alpha diversity (Chao1 index) of analysis of microbiota in the feces of mice (n = 3 to 5 per group). * : P<0.05, **: 0.01. (unpaired Mann–Whitney U-test). (b) The beta diversity through NMDS plot showing the difference in terms of OTU using Day 7 fecal samples. Bray-Curtis dissimilarities were ordinated by NMDS. (n = 3 to 5 per group). C.scindens TS: *C. scindens* KCTC 5591.

3. The characterization of specific bacterial taxa in the group with the CDI inhibitory effect

Although there was no difference in alpha and beta diversity among the groups, LEfSe analysis was performed to find bacterial taxa that differed significantly between groups. As a result, at 24 h after CDI, a significantly higher abundance of bacterial taxa was observed in each group. An analysis of all groups showed that there was a significant amount of *Ruminococcaceae* in the *L. plantarum* SNUG 10271 group (Fig. 5a). Also, *Ruminococcaceae* abundance was significantly higher in the *L. plantarum* SNUG 10271 group than the PBS group used as positive control (1:1) (Fig. 5b). Also, *Coprococcus* and *Clostridium* were also observed as significantly more abundant bacterial taxa in the *L. plantarum* SNUG 10271 group (Fig. 5b). By contrast, the *B. ovatus* SNUG 40239 group, which had the worst symptoms of CDI, had a significantly higher abundance of Bacteroidetes, Bacteroidia, Bacteroidales, *Bacteroidaceae*, and *Bacteroides* (Fig. 5b).

Next, all of the fecal microbiota sequencing data acquired from the CDI without group division was used to analyze the Spearman correlation between *C. difficile* and other bacterial taxa at the species level. As a result, *Clostridium*, *Ruminococcaceae*, *Coprococcus*, and

Akkermansia muciniphilia were identified as bacterial taxa that were negatively correlated with *C. difficile* (Table. 1). On the other hand, *Clostridium perfringens*, *Escherichia,* and *Enterococcus* were positively correlated with *C. difficile* (Table. 1).

As a result, it was observed that *Ruminococcaceae* and *Coprococcus* were biomarker taxa of the *L. plantarum* SNUG 40239 group and had a negative correlation with *C. difficile*.

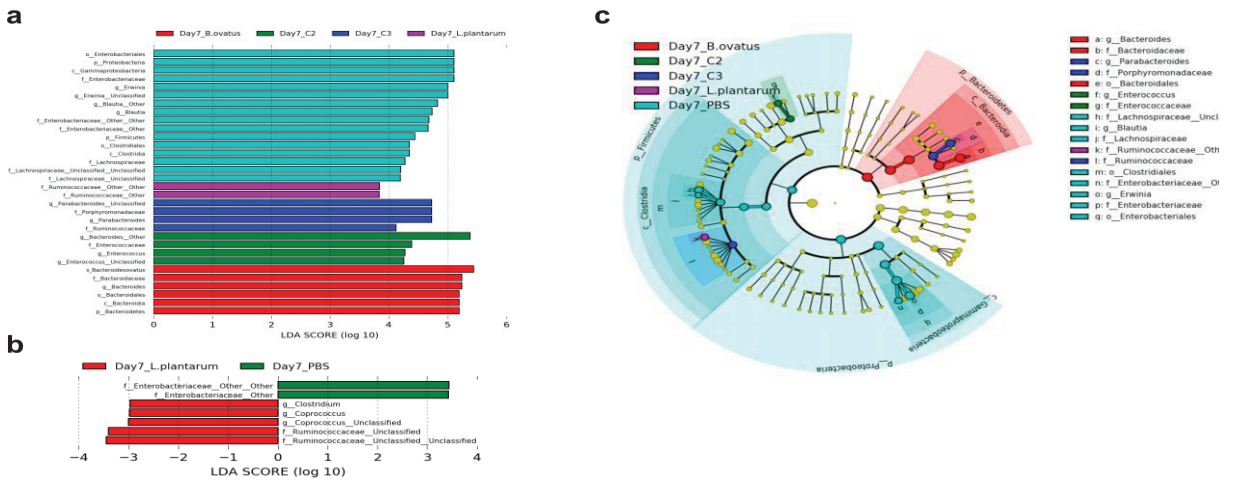

Figure 5. Comparison of dominant bacterial taxa at Day7

(a) LEfSe analysis showed the statistically significant difference taxa based on the abundance of taxa in each group. (b) The PBS group taxa are indicated with a positive LDA score (green), and taxa enriched in the *L. plantarum* SNUG 40239 have a negative score (red). Only taxa meeting an LDA significant threshold >2 are shown. (c) Cladogram using LEfSe method indicating the phylogenetic distribution of microbial community associated with administered inhibition strains. The brightness of point corresponds to its effect size. B.ovatus: *B. ovatus* SNUG 40239, C2: *C. scindens* SNUG 40402, C3: *C. scindens* KCTC 5591, L.plantarum: *L. plantarum* SNUG 10271. (p-value <0.05, LDA score > 2.0)

Taxon	p-value	Rho
Clostridium perfringens	$p < 0.001$	0.622
*Escherichia*_Unclassified	$p < 0.01$	0.512
*Clostridium*_Other	$p < 0.05$	-0.477
*Enterococcus*_Unclassified	$p < 0.05$	0.476
*Ruminococcaceae*_Other_Other	$p < 0.05$	-0.466
*Coprococcus*_Unclassified	$p < 0.05$	-0.430
Akkermansia muciniphila	$p < 0.05$	-0.412

Table 1. The results of the Spearman correlation of *C. difficile* abundance and other taxa abundances in which all groups at Day7.

We used the microbiota data of feces collected day 7 to analyze the Spearman correlation analysis between the abundance of *C. difficile* and other taxa. Only significant taxa were shown in the table ($P<0.05$).

4. CDI-related functional profile of gut microbiota

Previous microbiota analysis based on OTU showed no difference in beta diversity between groups. However, there were differences between the groups regarding the symptoms that followed infection. Also, certain bacterial taxa such as *Ruminococcaceae* and *Coprococcus* were present in different abundance. These results indicate that the intergroup microbiota was not the same, and we recognized the necessity of further analysis on microbiota at the metabolic functional level. The metabolic functional potential of each group was analyzed by PICRUSt based on the KEGG pathway and the results were used to observe beta diversity between the groups. Interestingly, each group was clustered (Fig. 6a). And the *L. plantarum* SNUG 10271 group with the best CDI inhibitory effect was located closest to the Non-infection group as a control (Fig. 6a). In addition, the secondary bile acid biosynthesis gene family was showed more tendency to be more in the *L. plantarum* SNUG 10271 group than the PBS group (Fig. 6b).

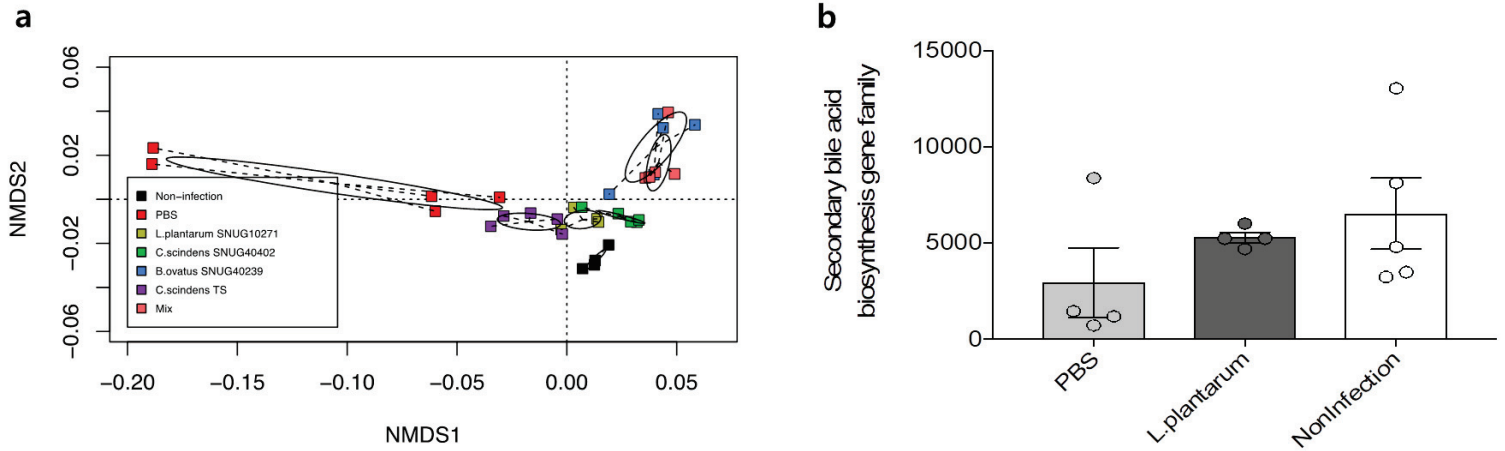

Figure 6. PICRUSt analysis to predict functions of gut microbiota

(a) The NMDS plot based on the functional profile of gut microbiota at Day 7. The dissimilarity between samples measured using Bray-Curtis method. (b) The comparison of secondary bile acid biosynthesis gene family acquired from PICRUSt analysis of each group at day 7. L.plantarum: *L. plantarum* SNUG 10271. C. scindens TS: *C. scindens* KCTC 5591.

5. The characterization of specific bacterial metabolites in the group with a CDI inhibitory effect

Due to the difference in beta diversity based on the PICRUSt analysis results, a metabolite analysis was conducted to find the metabolites associated with CDI inhibition. The PCA analysis was performed based on the statistically significant 11088 metabolites. As a result, a clustering phenomenon for individuals in each group was observed at Day7 (Fig. 7a). The *L. plantarum* SNUG 10271 with the best CDI inhibitory effect and the Non-infection groups were clustered to the upper left section (Fig. 7a). The PBS group and the *B. ovatus* SNUG 40239 group, which had the most severe symptoms of CDI, tended to cluster together (Fig. 7a). Also, the *C. scindens* SNUG 40402 groups and the *C. scidnens* KCTC 5591 group, which were more implicated in controls and *B. ovatus* SNUG 40239 on mortality, were observed to be clustered in the lower right section (Fig. 7a). The Mix group was distributed in both the *B. ovatus* SNUG 40239 group and the *L. plantarum* SNUG 10271 group (Fig. 7a).

Through PCA analysis, we were able to deduce the difference in metabolites between groups and found that the metabolites of the groups could be different. To find a biomarker metabolite in the *L.*

plantarum SNUG 10271 group, the PCA plot was converted into a loading plot and analyzed. As a result, we found that two metabolites that were present significantly high abundance only in the *L. plantarum* SNUG 10271 group and the Non-infection group; identification through MassLynx confirmed that the dimer and trimer of DCA formed during the ionization of two metabolites using ESI (Fig. 7b).

To verify whether DCA exists at a significantly high concentration in the *L. plantarum* SNUG 10271 group, we performed quantitative analysis in tofMRM mode using standard bile acids, TCA, CA, CDCA, LCA, and DCA. As a result, DCA was measured at significantly higher concentrations in the *L. plantarum* SNUG 10271 group compared with the PBS group, the *C. scindens* SNUG 40402 and the *B. ovatus* SNUG 40239 group after 24 h of CDI (Day8). In summary, DCA was determined to be a biomarker in the *L. plantarum* SNUG 10271 group, suggesting the CDI inhibitory effect is correlated with the significantly high concentration of DCA.

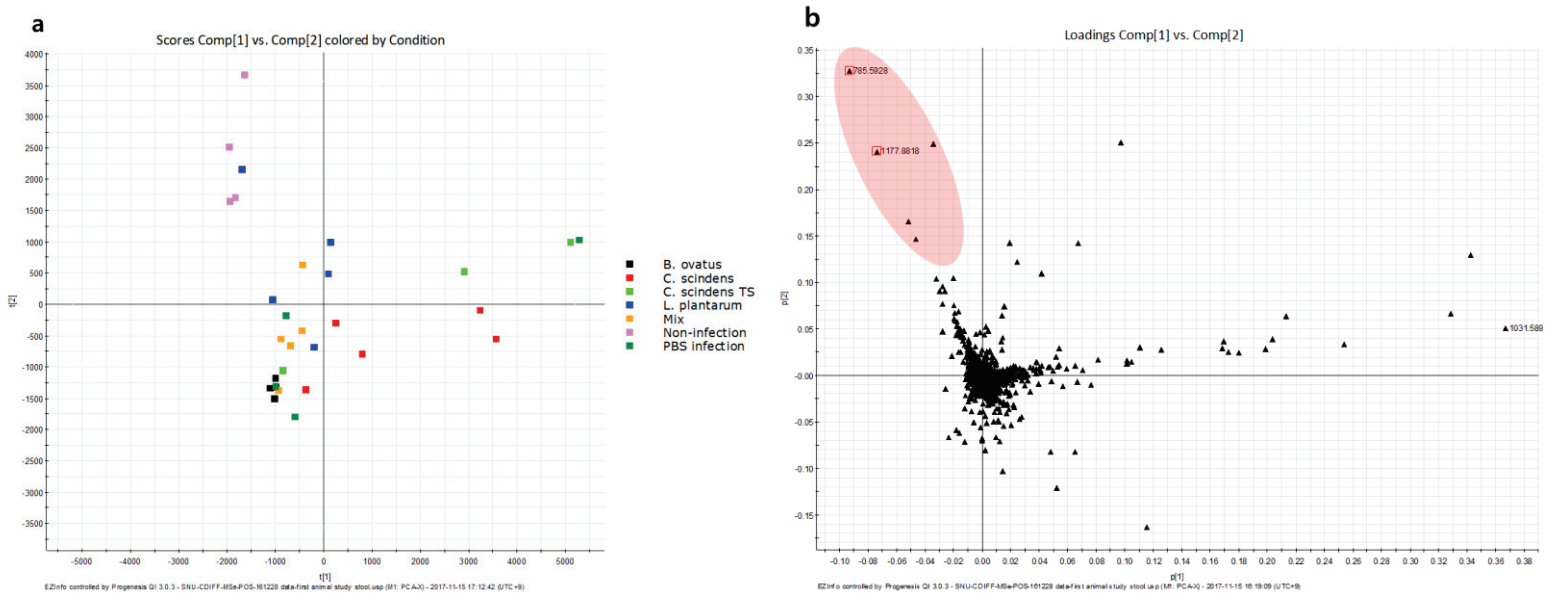

Figure 7. Gut metabolome analysis via Progenesis QI at Day 7.

(a) The PCA plot input with statistically significant 11088 metabolites among 26272 total metabolites (n = 3 to 5 per group). (b) The Loading plot for finding metabolites in the *L. plantarum* SNUG 10271 group. The red box was identified as DCA with a biomarker of the *L. plantarum* SNUG 10271 group. Metabolites included in the red circle are significantly high abundance only in the *L. plantarum* SNUG 10271 group and Non-infection groups. (n = 3 to 5 per group).

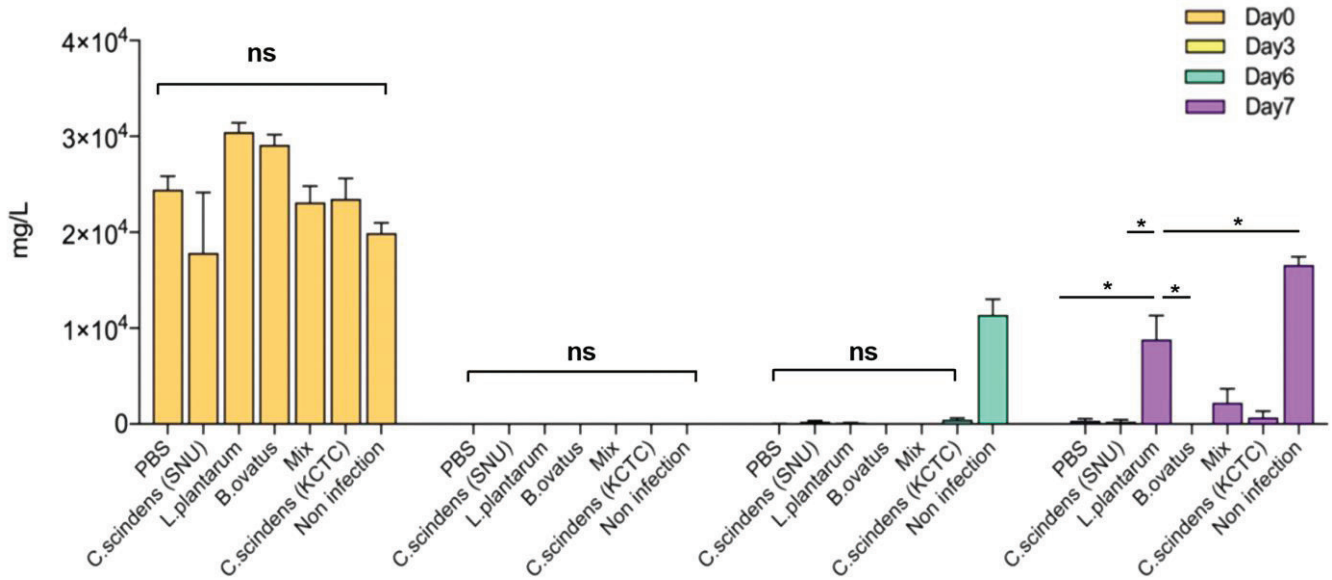

Figure 8. Concentration change of DCA over time.

The DCA profile in the feces of mice (n = 3 to 5 per group). Mean values ± s.e.m. are shown. *: P<0.05, **: P<0.01. (unpaired Mann–Whitney U-test).

6. The correlation of gut microbiota with bile acids.

We analyzed the correlation between DCA and gut microbiota for understanding the CDI inhibitory effects observed in the *L. plantarum* SNUG 10271 group. The Spearman correlation between the microbiota of the *L. plantarum* SNUG 10271 group at all time points and DCA concentration was analyzed. As a result, it was observed that *Ruminococcaceae* had the highest positive correlation with DCA (Fig. 9). *Coprococcus* and *Lachnospiraceae* were also observed to have a positive correlation (Fig. 9). Among the bacterial taxa with a positive correlation with DCA, *Clostridium orbiscindens* was specified to the species level (Fig. 9). On the other hand, *Akkermansia muciniphilia* and *Parabacteroides* were observed to be negatively correlated with DCA (Fig. 9).

Furthermore, the Spearman correlation between microbiota and other bile acids, such as TCA, CA, CDCA, DCA and LCA, concentrations associated with *C. difficile* spore germination and growth were analyzed without group discrimination at the post-infection time point (Day7). Consequently, it was observed that *Coprococcus* and *Ruminococcaceae* had a negative correlation with TCA, but a positive correlation with the secondary bile acids, DCA and

LCA (Fig. 10). On the other hand, *Lactobacillus* and *C. difficile* showed strong negative correlation with DCA and LCA (Fig. 10). In the case of *Prevotela* and *Bacteroidales*, it was observed that there was a high positive correlation with only the *C. difficile* spore germinant, TCA (Fig. 10).

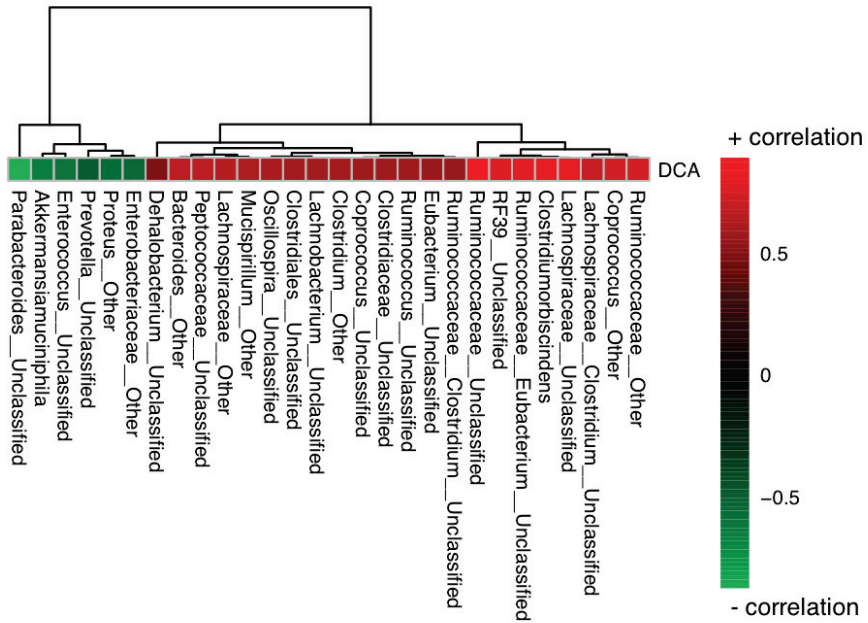

Figure 9. Spearman correlation of DCA concentration (mg/L) and microbial community in the *L. plantarum* SNUG 10271 group.

Correlation between Bacterial taxa and DCA in the *L. plantarum* SNUG 10271 group without time point distinction. Only significant taxa were shown ($P<0.05$).

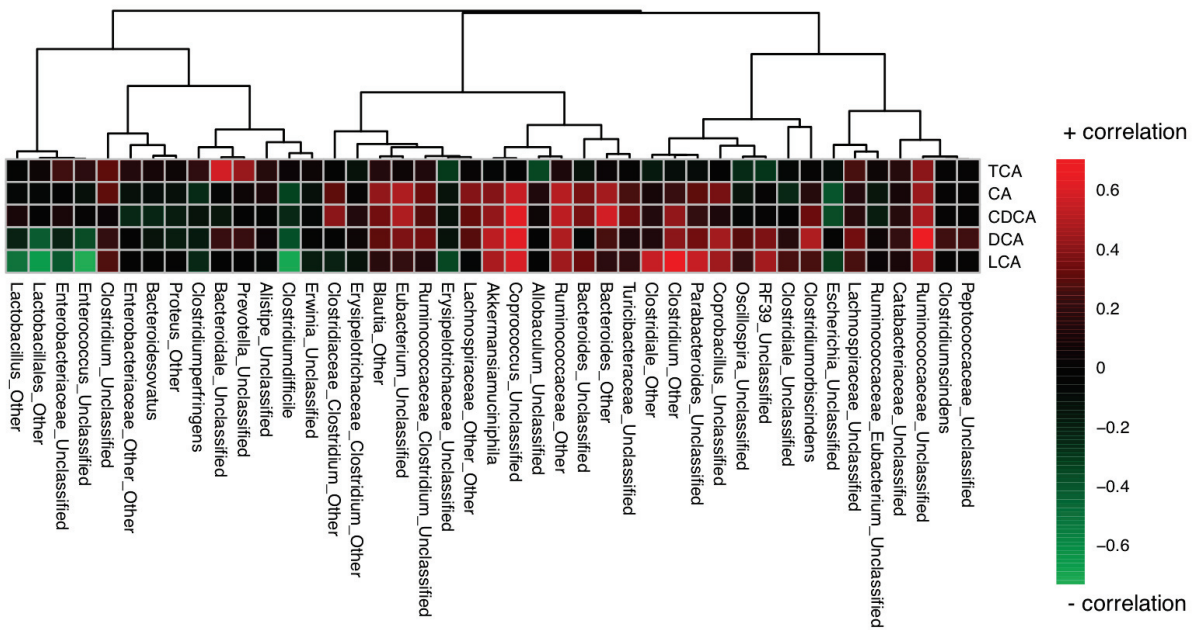

Figure 10. Spearman correlation between bile acids and microbial community.

Correlation between Bacterial taxa and bile acids without group distinction at Day7. Bile acids concentration was measured using feces collected at day 7. Only significant taxa were shown ($P<0.05$). TCA: taurodeoxycholic acid, CA: cholic acid, CDCA: chenodeoxycholic acid, DCA: deoxycholic acid, LCA: lithocholic acid.

IV. Discussion

The most important risk factor causing CDI is the use of various antibiotics [35]. Antibiotic use disrupts the healthy gut microbiota structure that prevents CDI in normal conditions [5]. For that reason, the goal of this study was to identify the strains with CDI inhibitory effects and to find the bacterial taxa and metabolites associated with inhibition by analyzing gut microbiota and gut metabolites. In this study, essential information for CDI prevention was found. Through experimentation, we have discovered the CDI inhibitory effect of *L. plantarum* SNUG 10271. In the process, DCA, which is known as a metabolite inhibiting *C. difficile* growth, was measured at a statistically significantly high concentration in the *L. plantarum* SNUG 10271 group. Also, we found the presence of the bacterial taxa *Ruminococcucea* and *Coprococcus*, which were not mentioned previously in relation to CDI.

The group administered *L. plantarum* SNUG 10271 showed a CDI inhibitory effect in this study. This inhibitory effect was assessed by measuring body weight change, mortality, *C. difficile* c.f.u., and toxin A&B titer. When compared to other groups, the *L. plantarum* SNUG 10271 group had a statistically significant inhibitory effect on

body weight loss and mortality. Body weight loss is considered to be significant, meaning that the average weight loss was reduced to within 7%. According to the prior studies, the weight loss of the PBS group as a positive control is around 10% [18], [36]. By contrast, this experiment showed that the body weight of the PBS group decreased by 19.8% at 72 h of infection. Based on this aspect, it can be seen that the inhibitory effect of *L. plantarum* SNUG 10271 on CDI is quite effective.

In addition, interesting results were observed in weight changes. In the *B. ovatus* SNUG 40239 group, the mean value of weight loss was greater than that of the PBS group as a control, and the mortality rate was one more death than the PBS group. Whereas, both the weight loss and mortality of the *L. plantarum* SNUG 10271 group indicated that *L. plantarum* SNUG 10271 was more effective at inhibiting CDI than the PBS group. These results suggest that the use of a single bacterial strain could control the severity of CDI symptoms.

While the *L. plantarum* SNUG 10271 group showed a significant effect on weight loss and mortality, there was no statistically significant difference detected in the *C. difficile* c.f.u. and the toxin A&B titer. Two reasons for this may be considered. First, there may be

cases where the process of toxin action on cells is inhibited. Toxin should be bound to the intestinal epithelial cell surface to induce symptoms [37]. However, such binding may be inhibited by secondary metabolites produced by gut microbiota. The secondary metabolites from *L. plantarum* SNUG 10271 could inhibit the cell binding of the toxins that lead to the symptoms. Therefore, this possibility cannot be totally dispensed with. Second, there may be errors resulting from the stool weighing process. CDI mice begin to show severe diarrhea symptoms beginning at 36 h of infection. These diarrhea symptoms lasted up to 96 h after infection. At the time point where the symptoms are most severe, the GI tract of the infected mouse was observed to be empty. The individuals whose fecal samples were measured for *C. difficile* c.f.u. and toxin A&B also had severe diarrhea symptoms. Thus, a large proportion of the collected feces contained transparent liquids such as mucus. This was reflected in the weighing of the feces, and an accurate weighing of the part containing the bacteria was not carried out, which may have affected the *C. difficile* c.f.u. and toxin A&B titer results. In fact, in some papers, the *C. difficile* c.f.u. and toxin A&B titer is measured using cecum content at 24 and 48 h, when symptoms become severe [18], [38], [39]. In this case, it is difficult to observe the

symptoms after CDI. In our study, when comparing the pellets of feces that had met the minimum weight and the pellets weighing more than the minimum weight, it was observed that the mass of the pellet was larger in the second case than for the first. Given these considerations, the failure of the *C. difficile* c.f.u. and toxin A&B titer to be consistent with weight change and mortality may be associated with the weighing error present in the feces weight measurement.

The interesting result of this experiment was observed in the beta diversity. When beta diversity was measured with OTU as the input data, clustering between groups was not observed. However, when beta diversity based on the functional profile of gut microbiota was measured, clustering between the groups was observed. This phenomenon can be interpreted based on two cases. The first is that the diversity of gut microbiota may be similar but different in each bacterial taxa level. In other words, although the number of bacterial taxa constituting the groups is similar, there could be a difference in the kind of each bacterial taxa constituting the groups. The differences between the kind of the bacterial taxa differ from the functional aspect. Actually, the metabolic function of the microbiota composed of different bacterial taxa may be similar [40]. The second reason is found

in the process of sequence matching the functional profile of gut microbiota via OTU. OTU identifies specific taxa through a process using QIIME. At this time, even if two OTUs are identified as belonging to the same taxa, the sequence of two OTUs may not be completely identical. The sequence differences between OTUs identified as the same genus or species can be interpreted as different functions in the process of predicting functions of microbial community through PICRUSt. Therefore, it can be considered that the small difference in sequences that exists in OTU is reflected in the functional prediction, and as a result, it is considered that the phenomenon of each clustered group was observed.

The functions of microbial community produced with PICRUSt indicates that there was a difference in metabolic functions between groups. The same phenomenon was observed when PCA analysis was performed by measuring actual metabolites. There was, however, a difference between groups that were clustered together. This phenomenon is considered to be the gap between the predicted score and actual metabolic activity. Metabolites, which were seen as markers in the *L. plantarum* SNUG 10271 group, were identified by DCA, and a large amount of DCA concentration was measured in the *L. plantarum*

SNUG 10271 group.

However, there are some things which need to be considered. First, *L. plantarum* SNUG 10271 does not have complete gene sets that relate the secondary bile acid DCA. Nevertheless, in the group given *L. plantarum* SNUG 10271, a significantly higher concentration of DCA was measured after CDI. Namely, the increase in DCA concentration is the output of the changes caused by *L. plantarum* SNUG 10271, and in this experiment, this change is considered to have increased *Ruminococcaceae* and *Coprococcus* abundancy. The next point to consider is that the *C. difficile* c.f.u. did not decrease even though the concentration of DCA increased. However, as mentioned previously in this section, this may reflect a measurement error in the process of weighing the feces. Therefore, an additional experiment cohort is needed to confirm the *C. difficile* c.f.u. using cecum and to verify it more clearly.

We should also consider the presence of *Ruminococcaceae* and *Coprococcus*, which showed a positive correlation with DCA concentration and a negative correlation with *C. difficile* abundance. Both taxa have not been reported in relation to CDI in previous studies. However, since the taxa level currently identified is a family level and

genus level, it is considered important to classify these taxa to the species level. The reason that finding precise species in respect to *Ruminococcaceae* and *Coprococcus* is significant is that it showed that the change of abundance of some specific bacterial species may potentially affect CDI symptom inhibition even though there is no difference in alpha diversity and beta diversity.

Taken together, in this study, *Ruminococcaceae* and *Coprococcus* increased with the administration of the *L. plantarum* SNUG 10271 group, and an increase in DCA concentration was observed in the same group. As a result, mice in the *L. plantarum* SNUG 10271 group with this phenomenon showed CDI inhibition in regards to weight loss and mortality. Because this experiment requires a larger number of individuals to discuss statistical significance more reliably, additional iterative experiments are needed to confirm the effect of *L. plantarum* SNUG 10271 on CDI inhibition. Only the results of inhibition phenomenon, such as the effects on *Ruminococcaceae* and *Coprococcus* and DCA, have been shown. The mechanism of inhibition caused by *L. plantarum* SNUG 10271 is not completely understood. Therefore, it is necessary to clarify this mechanism in future studies.

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국문초록

장 내 균총에서 분리 한 *Lactobacillus plantarum* 의 *Clostridium difficile* 감염 저해 효과

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Clostridium difficile infection (CDI) 는 가장 널리 알려진 원내감염 중 하나이다. 항생제 복용으로 인한 장내 균총의 불균형이 가장 큰 CDI의 유해요인이다. 정상적인 장내 균총을 가진 사람들은 CDI에 상대적으로 잘 감염되지 않는다. 그렇기 때문에 건강한 장내 균총 중 CDI에 저항성을 갖는 특정한 세균을 찾기 위한 연구가 필요하다. 하지만 아직까지 장내 균총 중 CDI에 저항성을 갖는 세균에 대한 연구들이 미비한 상황이다. 따라서, 이번 연구는 건강한 사람으로부터 분리된 단일 균주들의 CDI에 대한 저해효과를 검증하고 그 기전을 찾는 것을 목적으로 하였다. 선행연구를 통해 *C. difficile* 생장 저해효과가 확인된 3개의 분리균주 (*L.plantarum* SNUG 10271, *B. ovatus* SNUG 40239, *C. scindens* SNUG 40402)를 선별하였다. 이번 연구에서 해당 균주의 생체내 CDI 저해효과를 동물실험을 통해 확인하였다. CDI 저해 효과 평가에는 감염 후 체중변화, *C. difficile* c.f.u., toxin A&B titer 측정결과가 사용되었다. 동물실험과정에서 수득한 분변은 장내 균총과 장내 대사체 변화 및 상태 분석에 사용되었다. 실험 결과,

L. plantarum SNUG 10271 이 투여된 그룹에서는 유의한 CDI저해 효과가 확인되었다. 장내 균총 분석 결과 *Ruminococcaceae*와 *Coprococcus*가 *C. difficile*과 음의 상관관계를 갖는 것이 확인되었다. 장내 대사체 분석결과 Deoxycholic acid (DCA)가 저해효과가 있는 개체들에서 높은 농도로 측정되었다. 이러한 결과를 통해 건강한 사람에게서 분리된 단일 균주의 CDI저해 가능성을 확인하였다. 또한, CDI 저해 기전을 이해하기 위해 필요한 *Ruminococcaceae*, *Coprococcus*, DCA와 같은 단서를 찾았다.

주요단어 : *Clostridium difficile* infection, 장내 균총, *Lactobacillus plantarum* SNUG 10271, *Ruminococcaceae*, *Coprococcus*, *Deoxycholic acid*

학번 : 2016-24052